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DNA BARCODING AND PHYLOGENETIC ANALYSIS OF *TYTO ALBA, OTUS BAKKAMOENA* AND *ATHENE BRAMA* FROM INDIAN SUBCONTINENT

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ABSTRACT

In this study we are reporting the DNA barcode sequence of COI gene for the 3 species of the order Strigiformes from Indian subcontinent. In this study we tested the efficacy of DNA barcode technique for the identification of road vehicle collision killed birds and submitted the DNA barcode of COI gene sequences for these species. Road vehicle collision killed bird tissue sample was collected in 95-100 % ethanol. Tissue samples was processed for DNA isolation, PCR and Sequencing. Folmers universal primers are used for the amplification of COI gene. We sequenced the COI gene from *Tyto alba, Otus bakkamoena* and *Athene brama* and compare it with the10 previously published similar sequences from the Genus *Tyto, Otus, Athene, Asio, Strix* and outgroup *Calotis versicolor*. Multiple sequence alignment and N-J phylogenetic tree construction was done. The DNA barcode technique significantly identified all the 3 species and molecular systematic study was performed. In this study we successfully applied the Folmers universal primers for the amplification of bird's specific COI gene and the DNA barcoding technique found suitable for the identification road kill avian fauna. COI gene DNA barcode for the three Indian birds species *Tyto alba, Otus bakkamoena* and Athene *brama* were generated and submitted to GenBank with accession number [GenBank : KR779892, GenBank : KR779893, GenBank : KR779894] respectively.

KEYWORDS: COI, DNA Barcode, India, Tyto Alba, Otus Bakkamoena, Athene Brama, NCBI, Road Kill

INTRODUCTION

In Indian subcontinent more than thirty species of Owls and Owlets are found. Owls and Owlets are known to play an important role in the bio-control of the pests. The population of these important species is declining rapidly as a result of wide spread degradation of forest, loss of habitat, use of rodenticides, use of pesticides in Agriculture and road collision. Owls and Owlets are the nocturnal raptors except few. Due to nocturnal creature it is difficult to identify the Owls and Owlet species just on the basis of morphological and photographic evidences and thus new technological solutions involving molecular methods are essential for accurate taxonomical classification and phylogenetic study. Barn Owl, Scops Owl and Spotted Owlet, widely distributed throughout the Indian subcontinent and abundant, especially, around human habitation (Ali & Repley 1983). These bird species are commonly found in Amravati district of Vidarbha region (Anon, 2009 ; Wadatkar *et al.* 2010). In this study we have first time carried out the molecular systematic of Barn Owl, Scops Owl and Spotted Owlet from the Indian subcontinent.

Common Barn Owl *Tyto alba,* A very pale, long- legged, midsized owl with odd heart – shaped white face with dark eyes. Paler than similar eastern Grass – owl : upperparts lack heavy brown markings, are speckled white and splashed

pale rufous , wigs and tail weakly barred, and under parts peppered with dark spots. Most birds are white below or have a slight buff tinge; a few (usually females) are brighter buff below. It is a found on nearly entire Subcontinent east from Pakistan, south of Himalayan foothills from Panjab through Nepal valley, east through Assam Valley , lower parts of S. Assam hills , C and S Bangladesh , and through Peninsula to Sri Lanka. It found Local and uncommon in mostly open non – desert habitats, often associated with man. It is normally nocturnal and roosts and nests mainly in cavities in old buildings or caves (Rasmussen, 2012)

Indian Scops Owl *Otus bakkamoena***,** A variable , usually dark eyed Scops owl with prominent black border to facial disk and sparsely but boldly marked plumage ; the dark outer margin on ear tufts, a nearly solid blackish crown between prominent pale supercilia and pale hind collar, fairly uniform upperparts with long back streaks and largely pale buff scapular. Bill tipped black; crown noticeably darker than mantle, and finer, longer streaks on upper and underparts (with fine cross barring below).It occurs on the entire region south of Himalayas and evidently co-occurs with *lettia in E.* Nepal terai. Common in forest, open woodlands, edge, scrub orchards, scattered trees, village gardens and shade trees. Habitat is strictly nocturnal, roosting in thick vegetation. Feed primarily on beetles and grasshoppers, but also eats geckos and occasionally small rodents. Calls mostly after dusk and before dawn. Spotted Owlets Athene *brama*, A very familiar small Owl with white spotted brown upperparts and brown scales on whitish underparts, heavy on breast. At close range, also note mottled leg feathering and slim legs with unfeathered toes. It occurs on nearly entire Subcontinent east from Pakistan and from base of Himalayas south through Peninsula and east to Assam valley, lower parts of S Assam hills and much of Bangladesh; up to 1600 m. Old listing only for Afghanistan. The most often seen owl owing to its abundance, habit of appearing before dark, and occurrence around dwellings and disturbed areas; typically roosts in family parties or pairs. Breeds Nov- Apr in cavities in trees, buildings, rock walls (Rasmussen, 2012)

The Owls and Owlets are the groups of animals belongs to the order Strigiformes. The Barn Owl belongs to family Tytonidae while Indian Scops Owl and Spotted Owlet belong to family Strigidae. Hence in this study total 1order, 2 family, 3 Genus and 3 species are considered. The tissue samples used in this study are obtained from the road vehicle collision study. No bird was harmed or killed in this study for sample collection.

DNA barcoding is the use of small nucleotide sequence from a standardised portion of mitochondrial genome for species identification and evolutionary studies. DNA barcoding uses the small sequence Cytochrome oxidase subunit I (COI) from mitochondria, and has the potential to discriminate the wide variety of plants and animals species. DNA Barcoding is considering a novel tool for the identification of species and for discovering the new species by using molecular methods. The 648 bp mitochondrial cytochrome c oxidase subunit I (COI) serve as fast and accurate marker for species identification by molecular methods (Savolainen *et al.* 2005; Hebert *et al.* 2004a, 2010) due to its high interspecific variation, low intraspecific variation, and relatively universal primers for taxonomic groups at the level of orders and even classes (Hebert *et al*. 2004a; Ward *et al*. 2005; Hajibabaei *et al.* 2006; Johnsen *et al*. 2010). By using this approach various animal groups have been studied such as Neotropical bats (Clare *et al.* 2007), North American birds (Kerr *et al.* 2007), Australian fishes (Ward *et al.* 2005), and tropical Lepidoptera (Hajibabaei *et al.*2006) and endangered species of bird such as Asisn Haubara Bustard (Arif *et al.* 2012). Species identification through DNA barcoding has many practical utilities such as in conservation biology (Neigel *et al.* 2007; Ward *et al.* 2008), food security control (Wong and Hanner 2008), and bird strike identification (Dove, 2008). The universal primers for this gene COI are very robust and enables the recovery of its 5' end in most of the animals phyla (Folmer *et al.* 1994). Birds are the most studied and taxonomically

variable class of animals and hence it is very much useful for testing the efficacy DNA barcoding in species identification.

Continued DNA barcoding of birds from different geographic regions of the world will be of great help in the process of delimiting species (Johnsen *et al.* 2010).

In the present study, we partially sequenced COI gene for the 3 species which include Barn owl (*Tyto alba)* , Indian Scops Owl (*Otus bakkamoena*) and Spotted Owlet (*Athene brama*) ,perform the blast analysis and comparison of the sequences of these species with other representative sequences from the Genbank database to construct the phylogenetic tree. In this work we are establishing the genetic barcode for 3 Indian birds' species. Until now very few DNA barcoding sequences of COI gene of Indian birds have been generated. This is the study in which DNA barcoding of two Indian Owls and one Owlet have been reported from Indian subcontinent. This data will be helpful to identify these species by molecular method, to study molecular diversity, forensic investigation, and application in illegal poaching and in conservation approach in a near future.

MATERIAL AND METHODS

Study area: The surveys were conducted on the major roads of the Amravati district for tissue sample collection from road killed birds. Amravati district is located (N $20^0$57.543, E$077^0$45.803) in the western part of the Vidarbha region of Maharashtra State, India. The study was carried out between Jan 2015 to June 2015. Amravati region experiences tropical climate with temperatures ranging between 13^0C and 22^0C during winter and between 23^0C and 45^0C during summer. The annual rainfall ranges between 1000mm and 2250mm.

Collection of Sample: The tissue samples used in this study are collected during a project on the road vehicle collision of birds. The tissue of the fresh road kills are collected in 95- 100 % ethanol, brought to laboratory and stored at -20^o c until use. No birds were harmed or killed during the study. This approach utilised because we would like to test the efficacy of this technique in the identification of road kills.

DNA Analysis

DNA Isolation and Polymerase Chain Reaction amplification of the COI gene:

DNA was isolated using QIAGEN kit, QIA amp DNA FFPE Tissue (CAT.NO. 56404) as per manufacturer's instructions. DNA was eluted in 20.0 µl of elution buffer. COI gene specific forward and reverse universal primers were used for the amplification are :

LCO- 1490 5'- GGTCAACAAATCATAAAGATATTGG- 3' and

HCO –2198 5'- TAAACTTCAGGGTGACCAAAAAATCA - 3'

PCR reaction mix was prepared for DNA samples. Final volume of each reaction was 25.0 µl. Thermal cycling program for PCR was used as below: Initial denaturation at 95 ^{o}C for 5 min, followed by 35 cycles of denaturation at 94^o C for 1 min, Annealing at 40 ^{o} C for 1 min, Extension at 72 ^{O}C for 1.30 min and final extension at 72 ^{o} C for 7 min and hold at 4 ^{o} c until use. Agarose gel electrophoresis of the PCR products was performed using 2% (w/v) agarose gel using standard 0.5X TBE gel electrophoresis buffer. Sizes of the amplicons generated by this primer pair is 708 bp and is also evident from the gel. The purification of all amplicons was performed using Purelink PCR product purification kit from Life technologies as per the manufacturer's instructions. The purified PCR products were again checked on 2% Agarose gel to

confirm that the amplicons are not lost during purification.

DNA Sequencing of PCR Products

DNA sequencing of PCR product was performed using both the primers using Applied Biosystems Big Dye Terminator V3.1 Cycle sequencing kit. The sequencing products were loaded on Applied Biosystems 3130 Genetic Analyzer (Applied Bio systems, Foster City, CA, USA). Sequences were analyzed using Sequencing Analysis 5.1 software available in the sequencing machine. These sequences were further copied and analysed using ChromasPro v 1.34. Forward and reverse sequences were aligned to form the contig with best sequence calls.

BLAST Analysis and Phylogenetic Tree Construction

Contig sequences of the 3 samples were further subjected to BLAST analysis using NCBI BLAST analysis tool. Representative sequences which show high sequence similarity from BLAST results were selected for multiple sequence alignment and for the construction of phylogenetic tree. These reference sequences (Table 1) and sample sequences was aligned multiple sequence alignment tool of Mega 6.0 software (Tamura *et al.* 2013). The alignment file was exported in the Mega format and Phylogenetic tree by Neighbor Joining method was drawn using MEGA 6.0 software (Tamura *et al.* 2013). Nonparametric bootstrapping was performed using 1000 replicates. To calculate the sequence divergence, the Kimura 2- parameter (K2P) was used.

RESULTS AND DISCUSSIONS

The partial mitochondrial COI gene sequence for *Tyto alba, Otus bakkamoena and Athene brama* are 677 bp , 642 bp and 650 bp in length respectively. All the 3 birds samples of the order Tytodiformes are successfully amplified by using the Folmer's Universal primers. The nucleotide sequences of the mitochondrial COI gene segment for the *Tyto alba, Otus bakkamoena* and *Athene brama* specimens have been deposited in the Gen Bank holding an accession number [GenBank : KR779892, GenBank : KR779893, GenBank : KR779894] respectively. The base statistics composition of the *Tyto alba, Otus bakkamoena* and *Athene brama* are represented in table 2.

The base composition analysis shows that approximately equal AT and GC content. The blast analysis was performed by using contig of 3 species of *Tyto alba, Otus bakkamoena,* and *Athene brama* under the study and it shows similarity respectively for Tyto alba [GenBank :EU410491] (99 %) , for Otus semitorques [GenBank :AB843644] (91 %) and for Athene brama [GenBank :KF961185] (99%). The Blast analysis for 3 species revealed identity in the range of 86 - 99 % for other species and the E- value for all the species was found 0.00.

Multiple sequence alignment of the sample sequences with the similar representative sequences from BLAST analysis (Table 1) was done by using Clustal W and phylogenetic tree was constructed using Neighbor – Joining method with 1000 bootstraps of replication. The evolutionary divergences between 13 nucleotide sequences is calculated by using the Kimura 2-parameter model (Kimura 1980). The number of base substitutions per site from between sequences are shown in table 3. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 609 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).The COI genetic distances for all 13 species varies in the range of 0.000 to 0.4250. The overall average genetic distance found is 0.2042236.

Phylogenetic Tree

The evolutionary history was inferred using the Neighbor-Joining method as shown in Fig. 1 (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The family Strigidae forms two separate lineages; out which one lineage represent the Genus *Otus* while second lineage represents the Genus *Athene, Asio and Strix*. All the species of both the lineages shows significant bootstrap support. Our sequence of *Tyto alba* shares a common node with the *Tyto alba* [GenBank : JN801468] of West Australia with high bootstrap score 99 in N-J tree. The second of our sample Indian scoop owl (*Otus bakkamoena*) form the separate lineage nearby to node with Otus semitorques with bootstrap score 100 in N-J tree. The third sequence of our submission, Indian Spotted owlet (*Athene brama*) share a common node with *Athene noctua* with high bootstrap support 95.

CONCLUSIONS

Birds are highly diverse group of vertebrates and it is best group to study the efficacy of DNA barcoding technique for species identification In this work we generated the DNA barcode sequence for 3 Indian birds species, Barn Owl, Scops Owl and Spotted Owlet from the Indian subcontinent belonging the order Strigiformes and submitted to GenBank. The DNA barcode technique successfully identifies and discriminates between the species. The fresh road kill tissue samples successfully utilised for DNA barcode generation. The phylogenetic analysis of the three species with representative sequences was done by using Neighbor- Joining method . The Universal primer for COI gene as proposed by Folmers *et al.* accurately amplify the COI gene in Avian species with high precision. This work will be utilised for species identification, phylogenetic analysis, to study molecular diversity and evolution, to curb poaching and most importantly for the conservation of these beautiful and useful avian fauna in a near future.

Competing Interest: The Author(s) declared that they have no competing interest.

Authors Contribution: ASR, GAW and JSW performed field survey and participated in sample collection. ASR and GAW design the study, carried out molecular biology experiment, perform the Bioinformatics analysis and draft the manuscript. All authors read and approved the final manuscript.

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Table 1: The Representative Sequences from Genbank Used for Construction of Phylogenetic Tree

Genbank Accession Number	Order	Family	Genus	Species	Number of base pair
JN801468	Strigiformes;	Tytonidae	Tyto	alba	679
KF432214	Strigiformes;	Tytonidae	Tyto	furcata pratincola	609
JQ287742	Strigiformes;	Tytonidae	Tyto	longimembris	683
AB842991	Strigiformes	Strigidae	Otus	semitorques	663
GQ482285	Strigiformes	Strigidae	Otus	lettia	694
EF515808	Strigiformes	Strigidae	Otus	lempiji	686
KF452076	Strigiformes	Strigidae	Athene	noctua	681
GU571634	Strigiformes	Strigidae	Strix	aluco	723
GQ922626	Strigiformes	Strigidae	Asio	flammeus	699
KC016068 (Outgroup)	Squamata	Agamidae	Calotes	versicolor	658

Table 2: Base Composition of COI Sequence for Three Species under Study

Species	T	C	A	G	Total	G+C Content	A+T Content
Tyto alba [GenBank : KR779892]	25.7	31.3	24.4	18.6	677.0	49.9	50.1
Otus bakkamoena [GenBank : KR779893]	24.8	34.7	23.5	17.0	642.0	51.7	48.3
Athene brama [GenBank : KR779894]	24.9	32.3	25.5	17.2	650.0	49.5	50.4

Table 3: Estimates of Evolutionary Divergence between Sequences Using Distance Matrix Analysis

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	Tyto_alba_KR779892													
2	Tyto_alba_JN801468	0.0100												
3	Tyto_furcata_pratincola_KF432214	0.0446	0.0446											
4	Tyto_longimembris_JQ287742	0.0945	0.0887	0.1043										
5	Otus_bakkamoena_KR779893	0.2143	0.2098	0.2188	0.2077									
6	Otus_semitorques_AB842991	0.2036	0.2013	0.2079	0.2170	0.1051								

7	Otus_lettia_GQ482285	0.2036	0.2013	0.2079	0.2217	0.1111	0.0083							
8	Otus_lempiji_EF515808	0.2036	0.2013	0.2079	0.2217	0.1111	0.0083	0.0000						
9	Athene_brama_KR779894	0.2408	0.2336	0.2310	0.2131	0.1807	0.1769	0.1813	0.1813					
10	Athene_noctua_KF452076	0.1993	0.1927	0.2059	0.1998	0.2088	0.1895	0.1940	0.1940	0.1339				
11	Strix_aluco_GU571634	0.1978	0.1956	0.2022	0.1977	0.1643	0.1607	0.1628	0.1628	0.1477	0.1765			
12	Asio_flammeus_GQ922626	0.2083	0.2106	0.2059	0.1712	0.1716	0.1597	0.1618	0.1618	0.1532	0.1639	0.1524		
13	Calotes_versicolor_KC016068	0.4014	0.3866	0.4132	0.3863	0.4031	0.4016	0.4016	0.4016	0.4207	0.4250	0.3882	0.3752	

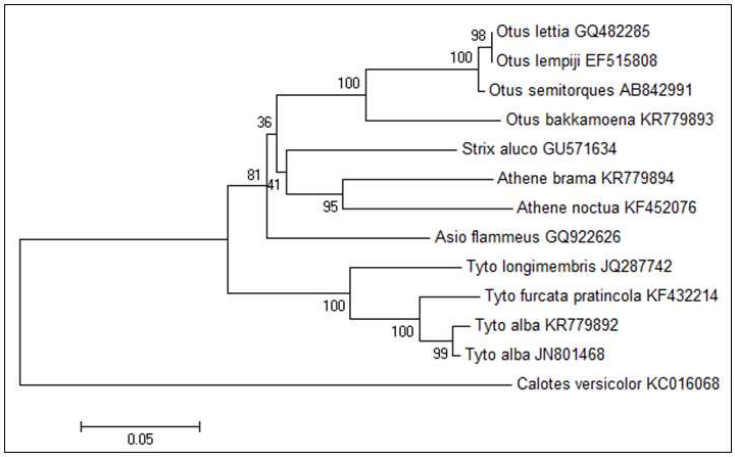

Figure 1: Neighbor Joining Phylogenetic Tree Showing the Evolutionary Relationship among the 13 Samples Including One Out-Group (*Calotes Versicolor* Kc016068). Numbers at the Respective Nodes are Percentage of 1000 Bootstrap Replicates. Bar Indicates Genetic Distance Due to Sequence Variation